HHS Public Access

Author manuscript

Neurobiol Aging. Author manuscript; available in PMC 2018 December 01.

Published in final edited form as:

Neurobiol Aging. 2017 December ; 60: 92–103. doi:10.1016/j.neurobiolaging.2017.08.010.

Targeted neurogenesis pathway-based gene analysis identifies ADORA2A associated with hippocampal volume in mild cognitive impairment and Alzheimer's disease

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[€]Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

Disclosure statement

The authors have no conflicts of interest to disclose.

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Abstract

Alzheimer's disease (AD) patients display hippocampal atrophy, memory impairment, and cognitive decline. New neurons are generated throughout adulthood in two regions of the brain implicated in AD, the dentate gyrus of the hippocampus and the sub-ventricular zone of the olfactory bulb. Disruption of this process contributes to neurodegenerative diseases including AD and many of the molecular players in AD are also modulators of adult neurogenesis. However, the genetic mechanisms underlying adult neurogenesis in AD have been underexplored. To address this gap, we performed a gene-based association analysis in cognitively normal and impaired participants using neurogenesis pathway-related candidate genes curated from existing databases, literature mining, and large-scale genome-wide association study findings. A gene-based association analysis identified *ADORA2A* as significantly associated with hippocampal volume and the association between rs9608282 within *ADORA2A* and hippocampal volume was replicated in the meta-analysis after multiple comparison adjustments (p=7.88×10^{-6}). The minor allele of rs9608282 in *ADORA2A* is associated with larger hippocampal volumes and better memory.

Keywords

Neurogenesis; ADORA2A; hippocampal volume; memory; Alzheimer's Disease; gene-based association analysis; NMDA-receptor antagonist

1. Introduction

Adult neurogenesis occurs throughout life in specific regions of the brain in humans. In rodents, neural stem cells differentiate to new neurons in several regions of the brain, but studies show that adult neurogenesis is limited to the dentate gyrus (DG) of the hippocampus and the sub-ventricular zone of the olfactory bulb in humans (Eriksson, Perfilieva et al. 1998, Spalding, Bergmann et al. 2013, Bond, Ming et al. 2015). The hippocampus is the most important region of the brain for new learning and episodic/spatial memory. The new neurons generated during adult neurogenesis are incorporated into hippocampal network circuitry during construction and maintenance of neural circuits and contribute to learning and memory (Aimone, Li et al. 2014, Winner and Winkler 2015). The progenitor cells in DG divide periodically, and DG experiences stability in neurogenesis throughout life (Scharfman and Bernstein 2015, Horgusluoglu, Nudelman et al. 2016). In rodents, neural stem cells (NSCs) in the DG make approximately 8,000 to 10,000 new neurons per day. However, the proportion of hippocampal neurogenesis decline in human is smaller than mice with aging (Amrein, Isler et al. 2011, Spalding, Bergmann et al. 2013). In 1998, the presence of adult-born neurons in the dentate gyrus of the human hippocampus had been identified by using cancer patients who had received the labelled 5-bromo-2'-deoxyuridine (BrdU) in hippocampal neurons (Eriksson, Perfilieva et al. 1998). In adults, the annual turnover of stem

cells into neurons is 1.75% with a modest decline during aging (Spalding, Bergmann et al. 2013). By contrast, the estimated annualized hippocampal atrophy rate is 1.41% per year for cognitively normal older people and 4.66% for patients with AD pathology (Barnes, Bartlett et al. 2009). Disruption of adult neurogenesis process has been postulated to contribute to neurodegenerative diseases including AD. Alterations in hippocampal neurogenesis in AD could either provide protection by proliferation of neural progenitor cells or cause accelerated neural degeneration due to impairment of neuronal networks and synaptic plasticity. Several studies in mice have combined structural MRI and histological approaches to investigate newborn neurons and neural stem/progenitor cells in neurogenesis-related brain regions and found that neurogenesis was associated with increased gray matter volume (Biedermann, Fuss et al. 2016). The relationship between hippocampal volume and adult neurogenesis in the human brain has not been studied yet.

Many molecular mechanisms and pathways play a role in the hippocampal neurogenesis process, including the proliferation of neural progenitor cells, the differentiation, migration, and maturation of adult neurons (Urban and Guillemot 2014, Horgusluoglu, Nudelman et al. 2016). Known modulators of adult neurogenesis include signaling transduction, vascular and immune systems, metabolic factors, and epigenetic regulation (Urban and Guillemot 2014, Borsini, Zunszain et al. 2015, Horgusluoglu, Nudelman et al. 2016). In particular, multiple factors such as neurotrophic factors, transcription factors, and cell cycle regulators control NSC proliferation, maintenance in neurogenic niche, and differentiation into mature neurons; these factors play role in networks of signaling molecules that influence each other during construction of neural circuits, and contribute to learning and memory (Fig. 1).

Disruption of the neurogenesis process has been postulated to contribute to neurodegenerative diseases including AD (Marlatt and Lucassen 2010). However, the mechanisms by which AD pathology affects neurogenesis are not completely understood. Alterations in the early stages of AD, such as amyloid-β deposition and inflammation, may impair the maturation of newborn neurons and inhibit hippocampal neurogenesis (Mu and Gage 2011). Genetic changes in neurogenesis-related pathways and genes may also play important roles in the alteration of NSCs maturation into newborn neurons (Horgusluoglu, Nudelman et al. 2016). Pathway- or gene-based association analysis has been used to study a number of complex neurodegenerative diseases, including AD, using a wide variety of phenotypes, including cerebrospinal fluid Aβ1–42 peptide level (Han, Schellenberg et al. 2010, Kim, Swaminathan et al. 2011), cerebral amyloid deposition (Swaminathan, Shen et al. 2012), brain glutamate levels (Baranzini, Srinivasan et al. 2010), and episodic memory (Ramanan, Kim et al. 2012). However, no study to date has evaluated the association between candidate neurogenesis-related genes and hippocampal volume. Thus, the goal of the present study was to perform a gene-based association analysis of neurogenesis pathway-related candidate genes in cognitively normal and impaired participants from ADNI cohort. Identification of genes that play a role in both hippocampal neurogenesis and AD may hold great promise for better understanding the role of neurogenesis in AD, as well as to aid in discovery of novel therapeutic targets for AD.

We used well-characterized participants from extensively studied cohort Alzheimer's Diasease Neuroimaging Initiatives (ADNI), which uniquely have GWAS data sets on the

same participants as well as multi-modal structural and functional neuroimaging (MRI, PET) data. A quantitative phenotype approach to genetic association studies provides the advantage of increased power sizes to detect significant genetic effects as compared to a traditional case-control design. We used hippocampal volume as a quantitative phenotype measured by MRI imaging, metabolic activity and amyloidosis in the hippocampus measured by PET imaging, and composite memory scores as quantitative traits to investigate that adult hippocampal neurogenesis-related genes and pathways are significantly associated with AD-related endophenotypes.

2. Materials and Methods

2.1. Participants

We used the participants of the Alzheimer's Disease Neuroimaging Initiative Phase 1 (ADNI-1) and its subsequent extensions (ADNI-GO/2) for this study. ADNI was launched in 2004 by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, the Food and Drug Administration (FDA), private pharmaceutical companies, and nonprofit organizations as a public-private partnership. The aim of ADNI has been to identify whether serial MRI, positron emission tomography (PET), sensitive and specific other markers, and clinical and neuropsychological assessments could be combined to measure the progression of mild cognitive impairment (MCI) and early AD. Participants aged 55–90 in ADNI cohort include approximately 400 cognitively normal older individuals (CN), 100 individuals with significant memory concerns (SMC), 800 individuals diagnosed with MCI, and 300 individuals diagnosed with AD. Clinical and neuroimaging procedures and the other information about the ADNI cohort can be found at http://www.adni-info.org/.

After the initial analysis, a meta-analysis was conducted with ADNI (Nho, Corneveaux et al. 2013, Weiner, Veitch et al. 2013) and two independent datasets, including the AddNeuroMed study (N=218; 66 CN, 77 MCI, 76 AD) (Lovestone, Francis et al. 2009, Nho, Corneveaux et al. 2013), and the Indiana Memory and Aging Study (IMAS) study (N=59; 29 CN, 24 MCI, 6 AD) (Nho, Corneveaux et al. 2013).

Written informed consent was obtained from each participant and all protocols were approved by each participating study and site’s Institutional Review Board.

2.1.1. Subject selection—Only non-Hispanic Caucasian participants were selected for this analysis by genetic clustering with CEU (Utah residents with Northern and Western European ancestry from the CEPH collection) and TSI (Tuscans in Italy) populations using HapMap 3 genotype data and multidimensional scaling (MDS) analysis after performing standard quality control (QC) procedures for genetic markers and participants (Ramanan, Risacher et al. 2014). Overall, 1,563 non-Hispanic Caucasian participants were included, as their genome-wide association study (GWAS) data passed the above population stratification and all other standard QC procedures (Kim, Swaminathan et al. 2011). Demographic information is shown in Table 1 for these participants.

2.2. Identification of candidate genes

Candidate genes which control the turnover process of neural stem cells/precursors to new functional neurons during adult neurogenesis were manually curated using a pathway-based systems biology approach (Fig. 1). Genes from known modulators of adult neurogenesis include those involved in signaling transduction, vascular and immune system function, metabolic factors, and epigenetic regulation (Nho, Corneveaux et al. 2013, Funa and Sasahara 2014, Urban and Guillemot 2014, Yao and Jin 2014, Borsini, Zunszain et al. 2015). Pathway-based approaches were used to manually curate these hippocampal neurogenesis-related genes through a review of existing databases and literature mining, resulting in a final gene list (Fig. 2). Specifically, we identified hippocampal neurogenesis-related genes using four publicly available databases (GoGene, Qiagen RT^2 Profiler PCR Arrays, Gene Ontology (GO) with Gene Set Enrichment Analysis (GSEA), and Mammalian Adult Neurogenesis Gene Ontology (MANGO)) and literature mining.

GoGene contains high-quality manual annotation with high-throughput text mining from literature using ontology terms. This database includes associations between genes and gene-related terms for ten model organisms extracted from more than 18,000,000 PubMed entries that cover process, function, location of genes and their relationship with diseases and compounds (http://gopubmed.org/gogene) (Plake, Royer et al. 2009). We performed a search for GoGene term “adult neurogenesis” to identify neurogenesis-related genes.

Qiagen RT^2 Profiler PCR Arrays are the one of the most reliable tools to analyze the expression of genes related to specific pathways. The human Neurogenesis RT^2 Profiler™ PCR Array contains 84 genes, manually curated using literature mining, which are highly related to the process of neurogenesis, such as neural stem/progenitor cells proliferation, differentiation, migration, and maturation into newborn neurons (www.qiagen.com). Growth factors, inflammatory cytokines, cell adhesion molecules, and cell signaling genes involved in the neurogenesis process were also represented in this profiling array. We included all genes from this Neurogenesis array in our gene list.

DAVID (the Database for Annotation, Visualization, and Integrated Discovery) is a publicly available functional tool which includes annotations from Gene Ontology (GO). Gene Set Enrichment Analysis (GSEA) pathway annotations were downloaded from Molecular Signatures Database version 5.0 (http://software.broadinstitute.org/gsea/msigdb). This annotation data comprised a collection of GO. A GO annotation contains a GO term associated with a specific reference which expresses the target or analysis associated with a specific gene product. Each GO term belongs to molecular function (Naveh, Shahar et al.), cellular component (CC), or biological process (BP). “Neurogenesis” was used as the GO term in this analysis to identify neurogenesis related gene sets from the GSEA database.

We also used MANGO, which consists of 259 genes designed and curated by Overall et al. (Overall, Paszkowski-Rogacz et al. 2012). In MANGO, all genes are classified by their positive, negative, or neutral effect on hippocampal neurogenesis and thusly annotated. We used recently updated MANGO version 3.1 to annotate genes.

We identified 510 genes from GoGene, 84 genes from Neurogenesis RT^2 Profiler™ PCR Array, 259 genes from MANGO database, and 93 genes from GSEA/Molecular Signatures Database. We combined all genes related to neurogenesis from the four databases (N=748). Then, each gene related to hippocampal neurogenesis was annotated from relevant literature. Initial Pubmed search using the keywords “adult hippocampal neurogenesis” for papers published until 9/2016 included 2,717 articles. We used Pubmed to identify if all 748 genes are related to hippocampal neurogenesis from 2,717 articles. After literature mining, among 748 genes, only 401 genes were related to adult hippocampal neurogenesis.

Finally, since a key goal was to identify candidate genes playing a role in both adult hippocampal neurogenesis and AD pathology, we focused on hippocampal neurogenesis-related genes which are also implicated in AD. For this purpose, we identified AD-associated susceptibility genes using the AlzGene database (http://www.alzgene.org/), which provides a comprehensive meta-analysis of genes previously identified in various AD association studies, and large-scale AD GWAS results (N=680) (Lambert, Ibrahim-Verbaas et al. 2013). The AlzGene database consists of a comprehensive, unbiased, publicly available catalog of all genetic association studies in the field of AD, which was identified from published papers by pubMed search using keywords “alzheimer* AND (genet* OR associat*)”. The gene list in AlzGene represents a summary of promising AD candidate genes. We then compared this gene list to the 401 genes previously identified as involved in adult hippocampal neurogenesis to filter the lists to 81 common genes related to both hippocampal neurogenesis and AD. These 81 genes were used in the association analysis.

2.3. Endophenotypes

Pre-processed baseline 1.5T and 3T MRI scans from 1,563 participants were downloaded from ADNI public website (http://adni.loni.usc.edu). FreeSurfer version 5.1 was used to extract total hippocampal and hippocampal subfield volumes, as well as total intracranial volume (ICV) (Dale, Fischl et al. 1999, Fischl, Sereno et al. 1999, Risacher, Kim et al. 2013, Risacher, Kim et al. 2015). Total hippocampal volume, as well as selected adult neurogenesis-related subfield volumes (CA1, CA23, CA4, and DG) (N=1,563), were used as endophenotypes for the association analysis. In addition, we used a composite score of episodic memory (N=1,563) (Crane, Carle et al. 2012) and CSF total tau levels (N=1,112) as endophenotypes to further characterize neurodegeneration (Shaw, Vanderstichele et al. 2009, Shaw, Vanderstichele et al. 2011).

2.4. Genotyping and Quality Control

ADNI samples were genotyped using Human 610-Quad, HumanOmni Express, and HumanOmni 2.5M BeadChips. Sample and SNP quality control procedures of GWAS data such as SNP call rate < 95%, Hardy-Weinberg equilibrium test $p < 1 \times 10^{-6}$, and frequency filtering (MAF ≥ 5%) were performed (Purcell, Neale et al. 2007, Saykin, Shen et al. 2010, Hohman, Koran et al. 2014, Ramanan, Risacher et al. 2014). Imputation of un-genotyped SNPs was performed using MaCH (Markov Chain Haplotyping) software based on the 1000 Genomes Project as a reference panel (Howie, Fuchsberger et al. 2012).

2.5. Association Analysis and Meta-Analysis

SNPs from the 81 candidate genes were located in untranslated regions (Laussu, Khuong et al. 2014), 3′ UTR, 5′ UTR, coding regions, intronic regions, and regulatory regions (±20 kb of upstream and downstream regions). A gene-based association analysis of hippocampal neurogenesis pathway-related candidate genes was performed in an additive genetic model using a set-based test in Plink v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell, Neale et al. 2007). After frequency and genotyping pruning, 18407 SNPs remained from 81 genes. (Purcell, Neale et al. 2007). After frequency and genotyping pruning, 18407 SNPs remained from 81 genes. A mean test statistic for each SNP within a gene was computed to determine other SNPs in linkage disequilibrium (LD; i.e., $r^2>0.5$). A quantitative trait analysis (QT) was then performed with each SNP. For each gene (set), the top independent SNPs (i.e., not in LD; maximum of 5) were selected if their p-values were less than 0.05 in the QT analysis. The SNP with the smallest p-value was selected first; subsequent independent SNPs were selected in order of decreasing statistical significance. From these subsets of SNPs, the statistic for each set was calculated as the mean of these single SNP statistics. The analysis was performed to estimate the additive effect of the selected SNP minor allele on the phenotypic mean (Purcell, Neale et al. 2007, Swaminathan, Shen et al. 2012). Covariates included gender, age, years of education, ICV, MRI field strength (1.5T vs 3T) and diagnosis status. An empirical p-value (20,000 permutations) was reported for each gene. In the discovery sample (ADNI-1/GO/2), a conservative significance threshold ($p < 0.00061$) was used based on Bonferroni correction for 81 genes. We subsequently performed a meta-analysis for genes and SNPs associated with hippocampal volume using data from the ADNI-1/GO/2, AddNeuroMed, and IMAS cohorts. The gene-based meta-analysis was performed using the weighted z statistic test (Stouffer's weighted z statistic) as implemented in R, with weight accounting for the sample size of each cohort. For SNP-based meta-analysis, METAL was used with a fixed-effect inverse variance model (Willer, Li et al. 2010). For meta-analysis, effect sizes were provided by standardized β coefficients from linear regression.

We also evaluated the effect of the minor allele rs9608282-T on hippocampal volume and composite memory score after the participants, which included only those diagnosed with MCI or AD, were classified as either a Memantine user (NMDA (+)) or a Memantine non-user (NMDA (−)) using two-way analysis of covariance (ANCOVA) for continuous variables and a chi-square for categorical variables implemented in SPSS 23.0. In addition, two-way analysis of covariance (ANCOVA) was used to examine the effect of the minor allele (rs9608282-T) on hippocampal volume in both amyloid-negative and amyloid-positive participants (classified as positive or negative by either baseline [^{18}F]Florbetapir PET scans and/or CSF Aβ1–42 level).

3. Results

3.1. Gene-based and SNP-based analysis of mean volumes of hippocampus and hippocampal sub-regions

The manual gene/pathway curation for hippocampal neurogenesis yielded 18407 SNPs in 81 genes (Fig. 2). In the discovery sample, the gene-based association analysis showed that

APOE and *ADORA2A* were significantly associated with hippocampal volume after Bonferroni correction (p-value=5×10^{-5}; Table 2).

One SNP (rs9608282) upstream of *ADORA2A* was most significantly associated with total hippocampal volume and sum of the hippocampal sub-region volumes ($p = 1.14\times10^{-5}$ and 2.5×10^{-4}, respectively; Table 3). Specifically, participants with no copies of the minor allele (N=1,317; GG genotype) had a smaller mean hippocampal volume compared to participants with one copy of the minor allele (N=236; TG genotype) or participants with two copies of the minor allele (N=10; TT genotype).

For replication of our major significant SNP finding, we analyzed independent samples from the ADNI, AddNeuroMed IMAS cohorts. SNP-based meta-analysis of *ADORA2A* in three independent cohorts (ADNI1/GO/2, AddNeuroMed, and IMAS) identified that rs9608282-T in *ADORA2A* are significantly associated with hippocampal volume ($p = 0.000043$, N=1,840, Table 4; $p = 7.88\times10^{-6}$, N= 1,840, Table 5, respectively). In ADNI1/GO/2, AddNeuroMed cohorts except IMAS, rs9608282-T exhibited a positive direction of effect on hippocampal volume.

Following the SNP-based association analysis, we performed a post-hoc analysis to measure the interaction effect of *APOE* and the *ADORA2A* SNP on hippocampal volume. There was no evidence of epistasis, modeled as interaction between *APOE* ε4 status and the minor allele of rs9608282 ($p = 0.54$). However, *ADORA2A* rs9608282-T and *APOE* ε4 exhibited independent but opposite effects on hippocampal volume (Fig. 3A, 3B), with a comparable effect sizes between the *APOE* ε4 allele and the presence of at least one copy of the minor allele at rs9608282. Participants carrying at least one copy of the minor allele of the *ADORA2A* SNP have larger hippocampal volumes than those without the minor allele, even in participants with *APOE* ε4 (p=0.001; Fig. 3C). The positive effect of the *ADORA2A* rs9608282-T allele on hippocampal volume was seen in both amyloid-negative and amyloid-positive participants (classified as positive or negative by either baseline [^{18}F]Florbetapir PET scans and/or CSF Aβ1–42 level). Specifically, rs9608282-T was significantly associated with larger hippocampal volumes in Aβ negative (p-value = 0.027) and Aβ positive participants (p-value = 0.015, Fig. 4). In addition, the association of the rs9608282-T allele with hippocampal volume and neurogenesis-related to sub-regions independent of diagnosis suggests that this effect might be a global phenomenon.

Since previous studies have suggested that *ADORA2A* plays an important role controlling NMDA-dependent synaptic toxicity and memory impairment (Tebano, Martire et al. 2005, Yee, Singer et al. 2007, Sarantis, Tsiamaki et al. 2015), we examined the interaction of taking Memantine, a NMDA-receptor antagonist, and *ADORA2A* rs9608282 on hippocampal volume and memory performance. Participants diagnosed with MCI or AD were classified as either a Memantine user (NMDA+) or a Memantine non-user (NMDA−). We found that NMDA-participants carrying at least one copy of the minor allele (T) of the *ADORA2A* rs9608282 had a larger mean hippocampal volume ($p<0.001$; Fig. 5A). There was a significant interaction effect of NMDA-receptor antagonist use and *ADORA2A* rs9608282 on memory performance ($p = 0.009$). NMDA+ participants carrying two copies

of the major allele (G) of the *ADORA2A* rs9608282 had better memory performance (Fig. 5B).

3.2. Association of rs9608282 with episodic memory and CSF level of total tau

Given the association of *ADORA2A* rs9608282-T with larger hippocampal volume, we hypothesized that *ADORA2A* would also associated with episodic memory scores as they are highly related to hippocampal structure. As might be hypothesized, rs9608282-T was significantly associated with a better composite memory score (β=0.065; p=0.015) after controlling for age, gender, and years of education (Fig. 6).

A previous study suggested that hyperphosphorylated tau decreases adult neurogenesis in mouse model (Komuro, Xu et al. 2015). Therefore, we also assessed the effect of the rs9608282-T minor allele on CSF total tau level. As we hypothesized, rs9608282-T carriers showed decreased CSF total tau levels relative to non-carriers (β=−0.061; p=0.039), after controlling for age and gender (Fig. 7). However, there is no correlations of rs9608282 with CSF Aβ and phospho-tau levels.

Finally, we used gene expression data from the Allen Human Brain Atlas to evaluate if *ADORA2A* was expressed in neurogenesis-related regions in normal brains. *ADORA2A* was in fact highly expressed across the major adult neurogenesis related regions of the brain (Fig. 8) and was especially highly expressed in CA1 and CA2.

4. Discussion

We used well-characterized participants from the Alzheimer's Disease Neuroimaging Initiatives (ADNI), an extensively studied cohort that includes cognitively normal older individuals (CN), as well as participants with significant memory concerns (SMC), amnestic mild cognitive impairment (MCI), and Alzheimer's Disease (AD). Using targeted neurogenesis pathway-based gene analysis, we discovered a significant association of *ADORA2A* rs9608282-T with larger mean hippocampal volumes and volumes of neurogenesis-related hippocampal sub-regions, better episodic memory performance, and reduced CSF total tau. These findings suggested a protective effect of this SNP on brain structure and function in neurogenesis-related brain regions.

ADORA2A (Adenosine Receptor Subtype A2a) is a G-protein-coupled adenosine receptor that is involved with controlling synaptic plasticity in glutamatergic synapses (Cunha, Ferre et al. 2008, Krugel 2015). Previous work had indicated a physical and functional interaction of ADORA2A with dopamine D_2 receptors (Chen, Moratalla et al. 2001). However, A1R–A2AR heteromer controls the affinity of agonist binding to A2a receptors in the striatum and localizes in glutamatergic nerve terminals to control glutamate release (Ciruela, Casado et al. 2006). In addition to its abundance in the striatum, ADORA2A also plays an important role in hippocampus, particularly in neurogenesis in the CA3 region. A previous study demonstrated that inhibition of the A2a receptor induced synaptic damage in rat hippocampal nerve terminals (Cunha, Canas et al. 2006).

A reduction of A2a receptors in mice with traumatic brain injury has also been shown to decrease cognitive impairment (Ning, Yang et al. 2013). In fact, the adenosine 2a receptor localizes in microglial cells and may be a regulation of microglial function in response to brain damage (Cunha, Ferre et al. 2008). Since neuroinflammatory blockade is thought to enhance neural stem/progenitor cells activity and promote adult neurogenesis, A2a receptor-mediated control of neuroinflammation might be a vital mechanism in neurodegenerative diseases (Monje, Toda et al. 2003). Inhibition of the A2a receptor also prevents early Aβ-induced synaptotoxicity and memory dysfunction through a p38 MAPK-dependent pathway (Canas, Porciuncula et al. 2009), potentially suggesting additional roles for this receptor in AD. In fact, *ADORA2A* blockade prevented memory decline secondary to amyloid-beta accumulation, which is a major pathological hallmark in AD (Cunha, Canas et al. 2008). Another important role of ADORA2A is to modulate brain-derived neurotrophic factor (BDNF). Administration of an ADORA2A antagonist inhibits the actions of BDNF on GABA and glutamate release from the hippocampal nerve terminals (Vaz, Lerias et al. 2015). In addition, the A2a receptor is involved with control of N-methyl-D-aspartate (NMDA) receptor function by co-localizing with metabotropic glutamate 5 receptors (Glu5R) in hippocampal synapses (Tebano, Martire et al. 2005). The synaptic localization of A2a receptors plays a key role controlling NMDA-dependent synaptic transmission in the hippocampus (Rebola, Sachidhanandam et al. 2007). In fact, glutamate release is dependent on the activation of adenosine A2AR by endogenous adenosine (Canas, Porciuncula et al. 2009, Vaz, Lerias et al. 2015). Previous studies showed the relationship between NMDA receptor and Adenosine Receptor Subtype A2a, which supports our finding of a significant interaction effect of NMDA-receptor antagonist use and the *ADORA2A* rs9608282-T on memory performance.

rs9608282 is located upstream of *ADORA2A* (UCSC Genome Browser (GRCh37/hg19)) and is characterized by occurring during read-through transcription of two neighbor genes, *SPECC1L* (sperm antigen with calponin homology and coiled-coil domains 1-like) and *ADORA2A* (adenosine A2a receptor) on chromosome 22. This read-through transcription is a candidate for nonsense-mediated mRNA decay (NMD), which leads to no protein production. The inhibition of *ADORA2A* has been shown to enhance spatial memory and hippocampal plasticity through adult neurogenesis (Laurent, Burnouf et al. 2016). In the present study, rs9608282-T was associated with better memory and a larger hippocampal volume, suggesting that this variation may inhibit protein production of *ADORA2A*. Animal models or cell culture studies are needed to more completely characterize the function of this variation on brain structure and adult neurogenesis.

Based on gene expression data from postmortem human brains, the A2A receptor is highly expressed in neurogenesis-related regions (CA1, CA2, CA3 and dentate gyrus) of the hippocampus in the adult human brain. Since the dentate gyrus and CA3 regions are important for memory formation and pattern separation processes, as well as for learning new information, we believe the observed effect of the rs9608282-T variation may be protective for memory performance by altering neurogenesis in these regions. In addition, the association of the rs9608282-T allele with hippocampal volume and neurogenesis-related sub-regions independent of diagnosis suggests that this effect might be a global rather than AD-specific phenomenon. Consistent with protective effect of this variant, decreased CSF

total-tau protein levels were also observed in participants with at least one minor allele (T) of rs9608282.

Interestingly, *ADORA2A* rs9608282-T and *APOE* ε4 exhibit an independent but opposite effect on hippocampal volume. In sum, we observed a significant protective effect of a variant (rs9608282) in the neurogenesis-related *ADORA2A* gene on brain structure and function, including increased hippocampal volume, better memory performance, and reduced CSF tau. This finding suggests that the adenosine A2a receptor warrants further investigation as a potential target for future therapeutics to treat neurodegenerative disease and cognitive decline.

The eQTL analysis using the BRAINEAC brain tissue microarray-based gene expression database (http://www.braineac.org/) revealed that rs9608282 in *ADORA2A* is marginally associated with *ADORA2A* gene expression levels in the hippocampus (p-value = 0.172). Individuals carrying minor allele rs9608282-T have decreased expression levels in the hippocampus, showing a potential protective effect consistent with our SNP-based association results with hippocampal volume and memory. Increased *ADORA2A* levels lead to synaptic toxicity and memory impairment (Tebano, Martire et al. 2005, Yee, Singer et al. 2007, Sarantis, Tsiamaki et al. 2015).

The limitation of the present report is that even though we used three independent publicly available databases to identify a curated gene list related to adult neurogenesis, it is possible that we may have missed other neurogenesis related genes not represented in these databases. The other limitation is that even though a few studies combined MRI-based hippocampal volume with immunochemistry to reveal that there is a significant hippocampal atrophy and the reduction of hippocampal neurogenesis in animal models, it is still not clear if hippocampal atrophy is related to adult neurogenesis in humans due to lack of data sources. Another limitation for this study is the lack of replication in the gene-based analysis. In the AddNeuroMed and IMAS, *ADORA2A* did not show a significant association with hippocampal volume but showed a trend. After combining three independent cohorts, the meta-analysis result was significant due to the increased detection power. In addition, future studies are needed to identify functional evidence to validate this SNP in *ADORA2A*. However, the present findings support that the *ADORA2A* gene plays a role in adult neurogenesis. AD is associated with hippocampal volume loss the observed effects indicates the potential importance of further investigation of this gene in independent cohorts.

Acknowledgments

Data collection and sharing for this project was funded by the Alzheimer's Disease Neuroimaging Initiative (ADNI) (National Institutes of Health Grant U01 AG024904) and DOD ADNI (Department of Defense award number W81XWH-12-2-0012). ADNI is funded by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, and through generous contributions from the following: Alzheimer’s Association; Alzheimer’s Drug Discovery Foundation; BioClinica, Inc.; Biogen Idec Inc.; Bristol-Myers Squibb Company; Eisai Inc.; Elan Pharmaceuticals, Inc.; Eli Lilly and Company; F. Hoffmann-La Roche Ltd and its affiliated company Genentech, Inc.; GE Healthcare; Innogenetics, N.V.; IXICO Ltd.; Janssen Alzheimer Immunotherapy Research & Development, LLC.; Johnson & Johnson Pharmaceutical Research & Development LLC.; Medpace, Inc.; Merck & Co., Inc.; Meso Scale Diagnostics, LLC.; NeuroRx Research; Novartis Pharmaceuticals Corporation; Pfizer Inc.; Piramal Imaging; Servier; Synarc Inc.; and Takeda Pharmaceutical Company. The Canadian Institutes of Health Research is providing funds to support ADNI clinical sites in Canada. Private sector contributions are facilitated by

the Foundation for the National Institutes of Health (www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Disease Cooperative Study at the University of California, San Diego. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of Southern California. Samples from the National Cell Repository for AD (NCRAD), which receives government support under a cooperative agreement grant (U24 AG21886) awarded by the National Institute on Aging (AIG), were used in this study. Funding for the WGS was provided by the Alzheimer's Association and the Brin Wojcicki Foundation.

Additional support for data analysis was provided by NLM R01 LM012535, NIA R03 AG054936, NIA R01 AG19771, NIA P30 AG10133, NLM R01 LM011360, NSF IIS-1117335, DOD W81XWH-14-2-0151, NCAA 14132004, NIGMS P50GM115318, NCATS UL1 TR001108, NIA K01 AG049050, the Alzheimer's Association, the Indiana Clinical and Translational Science Institute, and the IU Health-IU School of Medicine Strategic Neuroscience Research Initiative.

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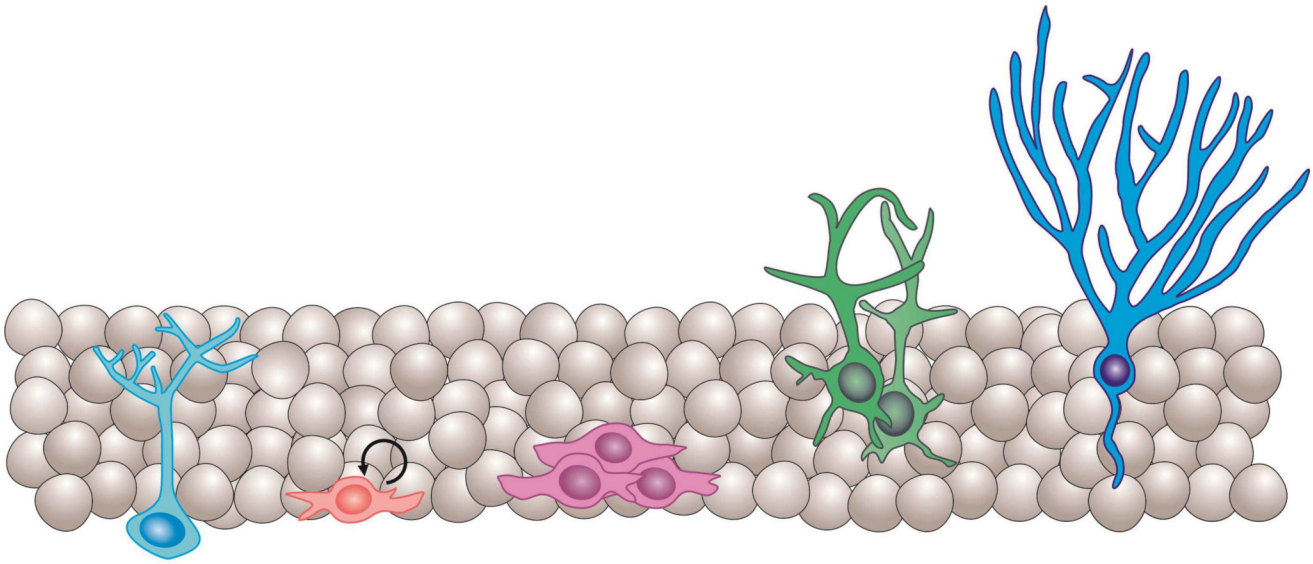
Highlights

- Candidate pathways and genes which play a role in neurogenesis in the adult brain are manually-curated.
- ADORA2A is significantly associated with hippocampal volume
- A SNP (rs9608282) upstream of *ADORA2A* is associated with larger hippocampal volume and better memory performance.
- rs9608282 may have a protective effect on brain structure and function in neurogenesis-related brain regions.
- There is a significant interaction effect of NMDA-receptor antagonist use and the *ADORA2A* rs9608282-T on memory performance.



Self-Renewal/Proliferation

ADAM10	ESR1	NOS3
ADRA2A	FGF1	PLCG1
APBB1	GRIN2B	PRNP
APP	GRN	PSEN1
BCL2	GSK3B	PTGS2
BDNF	HTR2A	S100B
CCL2	IGF1	SORL1
CHRNA7	IGF1R	TET1
CHRNB2	IL1B	TLR4
CNTF	LRRK2	TNF
CXCL1	NGF	VEGFA
DKK1	NOS1	

Differentiation/Migration

ADAM10	IFNG
APBB1	IGF1
APOE	IGF1R
BCHE	MEF2C
CAV1	MIF
CDK5	NEUROD1
CDK5R1	OLIG2
CXCL12	RELN
DYRK1A	S100B
FAS	SIRT1
HIF1A	

Maturation/Survival

ACHE	CHRNB2
ADORA2A	IL6
BCHE	MEF2C
CASP3	NOS1
CAV1	NPY
CCL2	S100B
CDK5	SLC6A4
CHAT	THRA

Figure 1. Genes playing roles in stem cells proliferation, differentiation, migration, and survival to new neurons during adult neurogenesis process

Glial-like radial stem cells (light blue); progenitors (pink); neuroblasts (purple); immature neurons (green); mature neurons (blue).

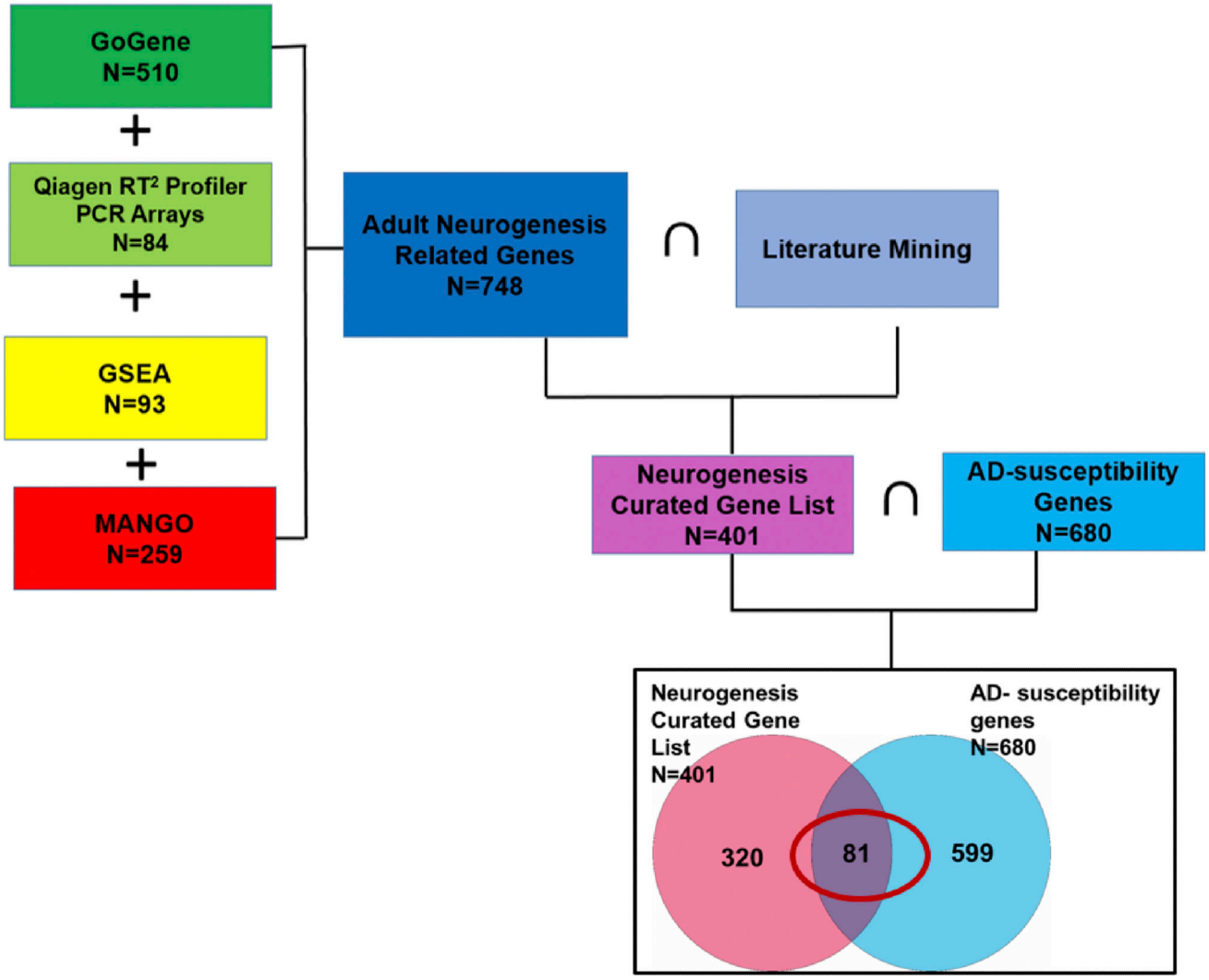


Figure 2. Venn diagram of Adult neurogenesis-related genes and AD-susceptibility genes

Manually curated neurogenesis and AD related gene. {(GoGene) ∪ (Mango) ∪ (Qiagen) ∪ (GSEA)} ∩ {Pubmed Mining} = 401. AD-susceptibility genes (N=680) from AlzGene database and large-scale GWAS results. Eighty-one common genes were identified associated with both neurogenesis and AD. ∪: Union; ∩: Intersection.

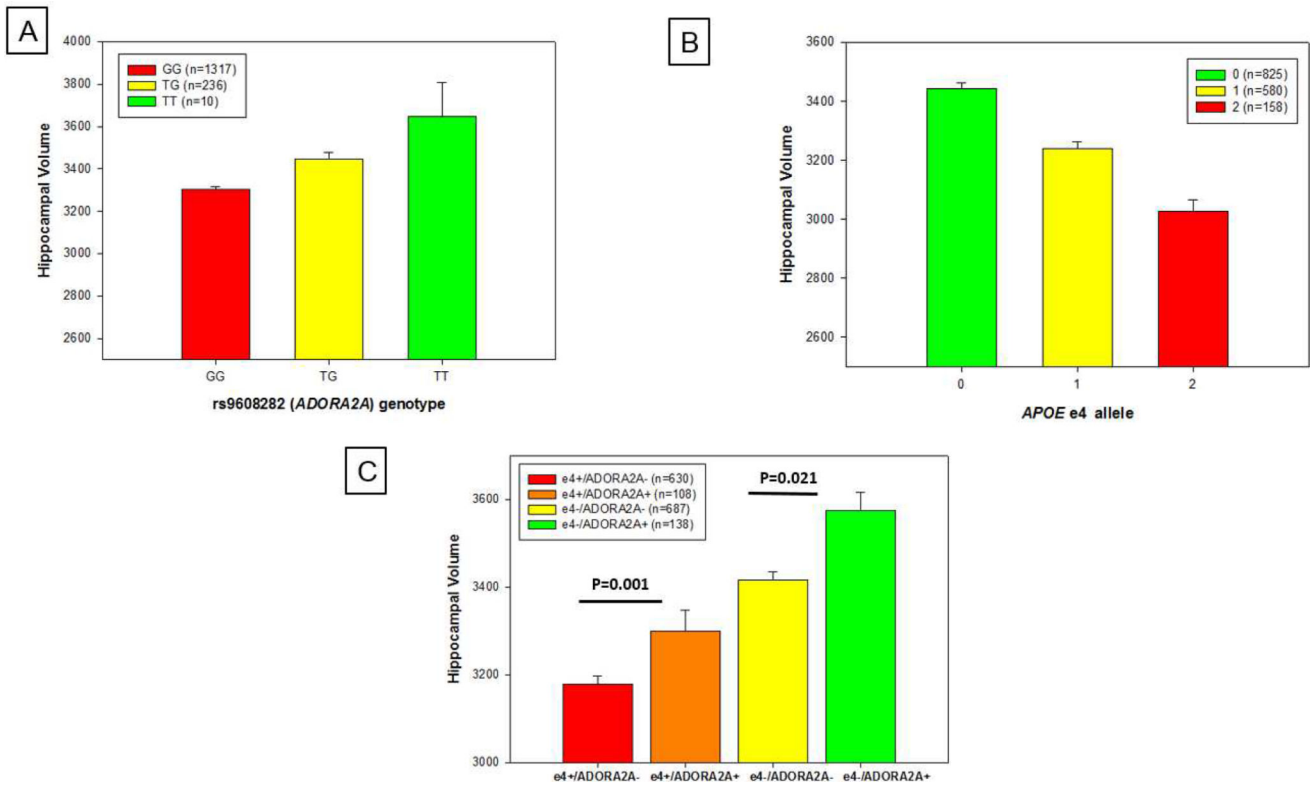


Figure 3. *APOE* ε4 and rs9608282 (*ADORA2A*) appear to exhibit independent, but opposite effect on hippocampal volume

Baseline hippocampal volume (adjusted for age, gender, ICV, MRI field strength) ± standard errors are shown based on (a) rs9608282 in *ADORA2A* (A2aR) across genotype groups. Presence of at least one copy of the minor allele (T) of rs9608282 was significantly associated with increased hippocampal volume ($p = 0.002$). Baseline hippocampal volume is also shown by (b) the number of *APOE* ε4 allele copies. Presence of at least one copy of the ε4 allele was significantly associated with decreased hippocampal volume ($p < 0.0001$). (c) For participants having *APOE* ε4 allele copies, participants carrying minor allele of rs9608282 had larger hippocampal volume than those who did not carry minor allele of the *ADORA2A* rs9608282 ($p = 0.001$).

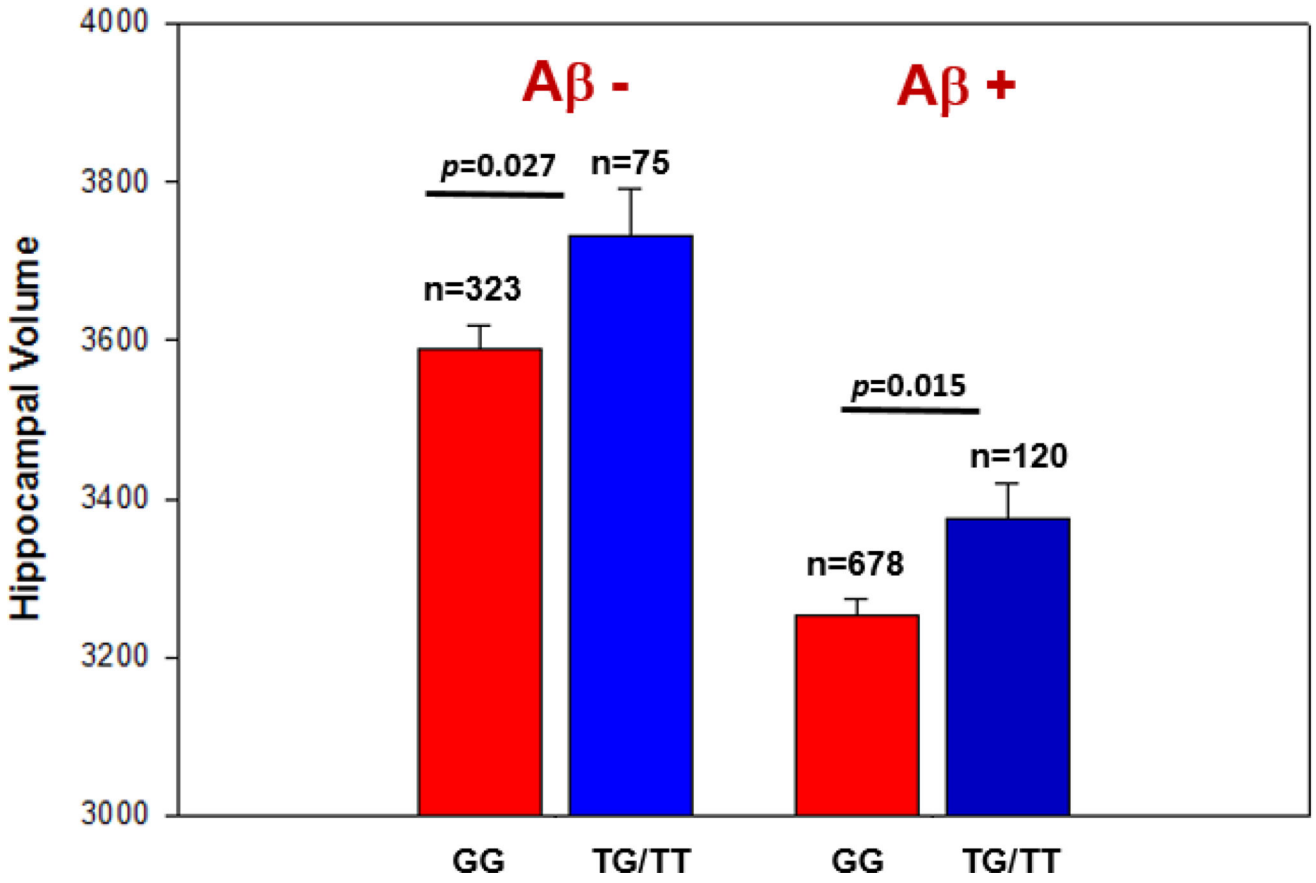


Figure 4. ***ADORA2A*** **rs9608282 is associated with larger hippocampal volume in amyloid-positive participants (classified by PET scan and/or CSF-amyloid beta level)**

For statistical analysis, each participant was classified by their amyloid status (positive versus negative) at the baseline visit (determined by standard cutoffs on [^{18}F]Florbetapir PET scan and/or CSF amyloid level). The effect of the T allele on hippocampal volume was present in both amyloid-negative (left column) and amyloid-positive (right column) participants. Upon statistical analysis, rs9608282 is significantly associated with hippocampal volume in amyloid-negative and even amyloid-positive participants (p = 0.027, p =0.015, respectively).

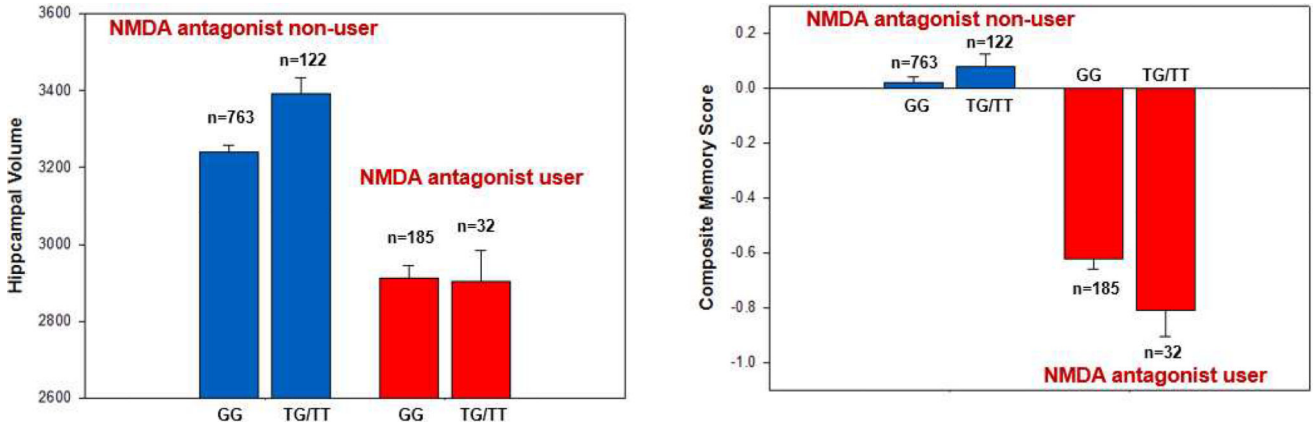


Figure 5. ***ADORA2A*** **rs9608282 is associated with larger hippocampal volume in Memantine non-users (NMDA (−)) and poorer memory performance in Memantine users (NMDA (+))**

For statistical analysis, cognitively impaired participants were classified as either a Memantine user (NMDA (+)) or a Memantine non-user (NMDA (−)). (A) The rs9608282 T allele was associated with a larger mean hippocampal volume in Memantine non-user participants ($p < 0.001$). (B) Participants carrying at least one copy of minor allele (T) of the *ADORA2A* rs9608282 variant and using a NMDA-receptor antagonist had poorer memory performance.

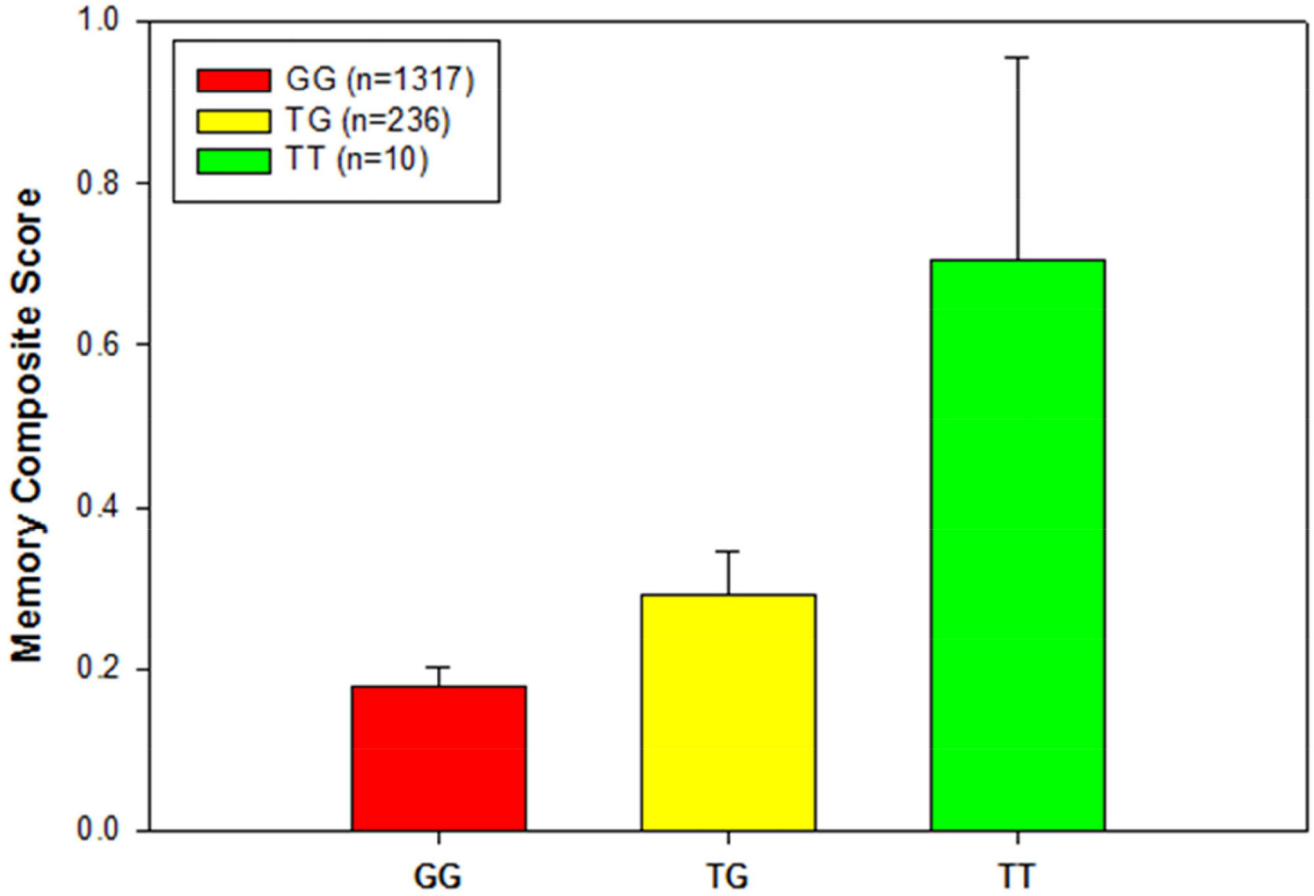


Figure 6. Association of memory composite score with rs9608282 in *ADORA2A* across genotype

Baseline memory composite score (adjusted for age, gender and education) ± standard errors are displayed based on rs9608282 genotype. Individuals with a TT genotype at the rs9608282 variant showed a 5% increase in memory performance relative to those with a GG genotype.

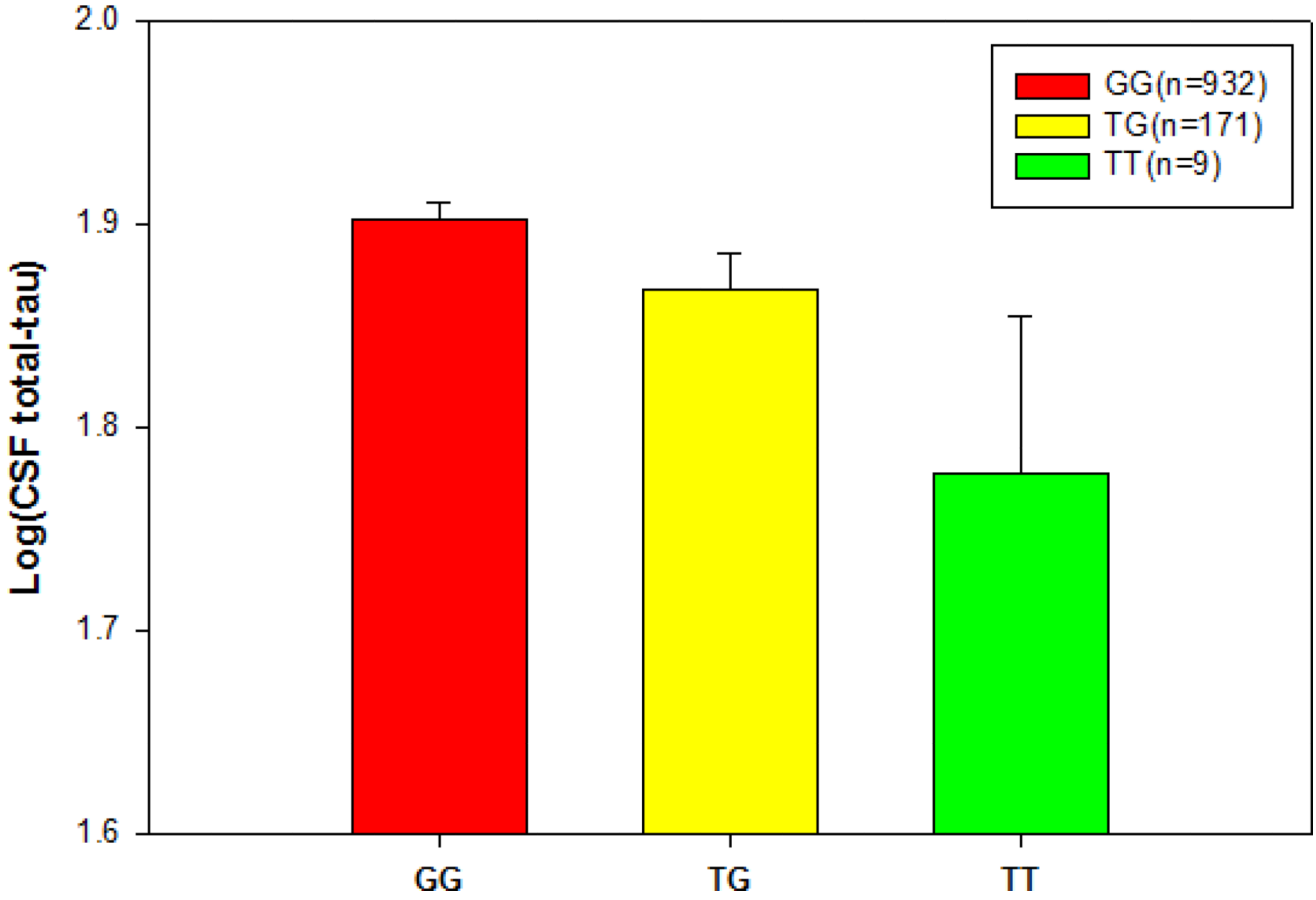


Figure 7.

Association of CSF tau level with rs9608282 in *ADORA2A* across genotype. CSF total tau level (adjusted for age and gender)) ± standard errors are displayed based on rs9608282 genotype. Individuals with a TT genotype at the rs9608282 variant showed significant decrease in CSF tau level relative to those with a GG genotype.

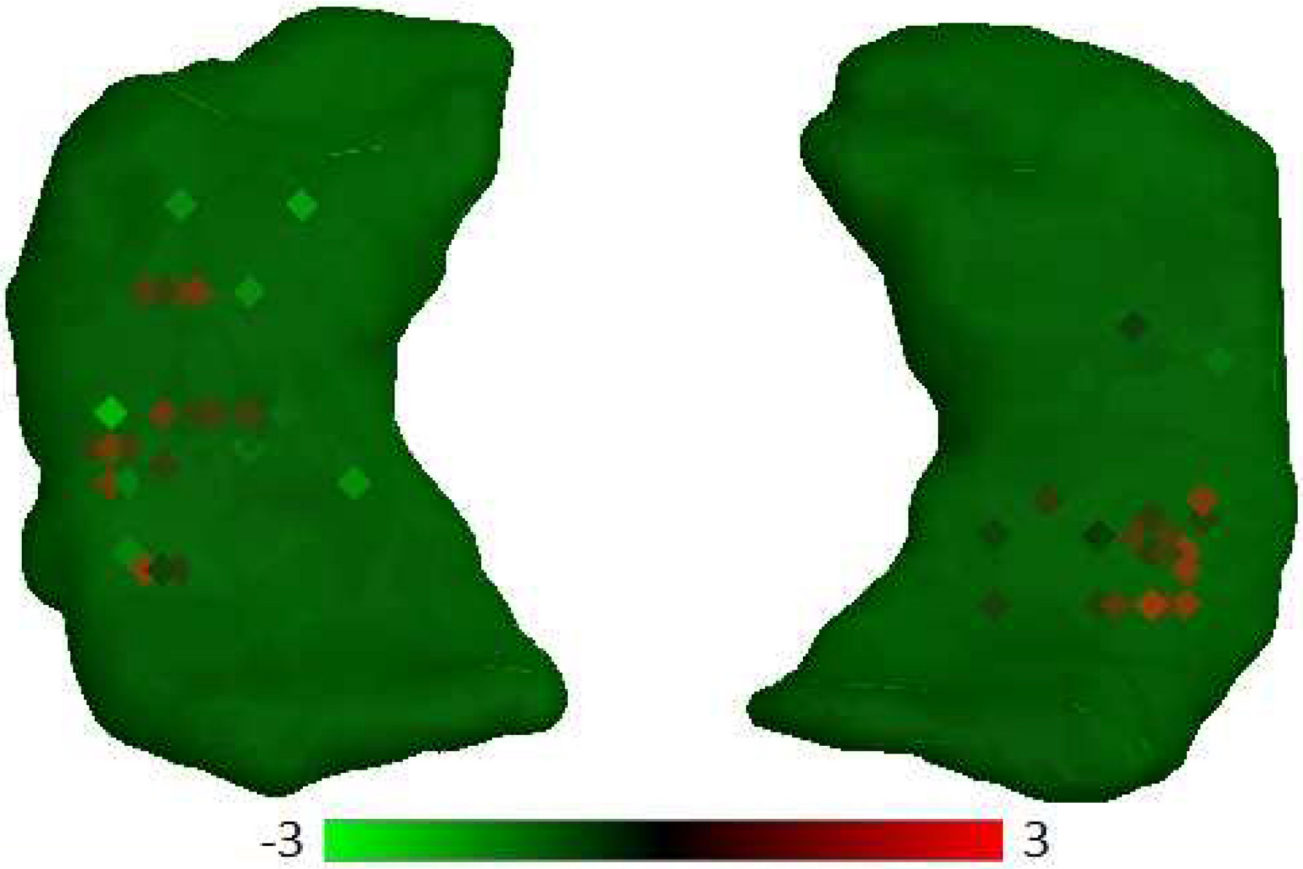


Figure 8. ***ADORA2A*** **expression profiles across the hippocampus region**

The square dot indicates the tissue sample location for human brain. In each square, heat map color represents the z-score over a probe ranging from green (z-score of −3 and below) through black to red (z-score of +3 and above). Red squares represents overexpression of the *ADORA2A* in specific locations, most especially in CA1 and CA2.

Table 1

Demographic and clinical characteristics of ADNI participants at the time of MRI scan

	CN	SMC	EMCI	LMCI	AD
N	367	94	280	512	310
Age	74.59 (5.57)	71.77 (5.65)	71.14 (7.26)	73.52 (7.65)	74.65 (7.79)
Gender (M/F)	192/175	38/56	158/122	318/194	176/134
Education	16.32 (2.68)	16.81 (2.57)	16.08 (2.67)	15.97 (2.91)	15.23 (2.97)
APOE (ε4−/ε4+)	267/99	62/32	160/119	232/280	104/206

Neurobiol Aging. Author manuscript; available in PMC 2018 December 01.

Table 2

81 genes of gene-based association results in the discovery sample for hippocampal volume using common variants (MAF ≥ 0.05) where empirical *p*-values were calculated using 20,000 permutations in PLINK

Gene	Number of SNPs in gene	Number of significant SNPs (p<0.05, r2<0.5)	Empirical gene-based p-value	List of significant SNP
ADORA2A	55	1	5×10^{-5}	rs9608282
APOE	85	5	5×10^{-5}	rs429358|rs7259620|rs34095326|rs4803770|rs157580
TLR4	58	2	0.01499	rs11789302|rs10759930
BCHE	201	4	0.02797	rs2686409|rs1355538|rs12107166|rs6807910
CXCL10	42	1	0.04795	rs4256246
S100B	220	1	0.05994	rs118078026
CXCL12	306	4	0.06194	rs11238990|rs11238991|rs1144472|rs17659345
TET1	387	3	0.06593	rs113716271|rs12776586|rs12221107
BCL2	368	5	0.07493	rs9957149|rs28564323|rs7236090|rs6567334|rs11872403
PTGS2	52	2	0.08492	rs7547677|rs2206593
ACHE	51	2	0.08791	rs13245899|rs73714210
SORL1	233	2	0.09091	rs9665907|rs643010
SLC6A4	63	1	0.09191	rs16965628
GRIN2B	1054	5	0.0999	rs34870448|rs11612709|rs12582848|rs11611667|rs2300256
NGFR	86	5	0.0999	rs584589|rs11466150|rs2072444|rs535717|rs2537710
MEF2C	232	1	0.1019	rs1065861
CXCL1	39	1	0.1039	rs2968710
NR3C1	238	5	0.1209	rs4912912|rs17209237|rs10050756|rs7719514|rs12653301
DKK1	40	2	0.1249	rs11001581|rs7100461
GRN	37	1	0.1319	rs114641762
CHAT	189	5	0.1379	rs885834|rs11101179|rs1720367|rs4615945|rs74981858
CHRNB2	39	5	0.1499	rs9616|rs9427094|rs12072348|rs67860750|rs4845653
Syn3	1388	5	0.1718	rs2157188|rs9609643|rs2710348|rs180958069|rs5749521
CCL2	43	1	0.1758	rs111843487
IGF1	89	4	0.1888	rs1549593|rs10860862|rs12821878|rs80280982
VEGFA	59	3	0.1908	rs9381248|rs3025006|rs699946
NGF	135	2	0.1978	rs6537860|rs4320778
MIF	77	1	0.2008	rs738807
IL6	77	2	0.2338	rs2069840|rs62449498
CASP3	94	2	0.2378	rs4647634|rs2696059
MMP9	57	2	0.2537	rs73112805|rs3918253
PRNP	94	2	0.2747	rs6052766|rs67017873
SNCA	444	1	0.2927	rs187644542

Gene	Number of SNPs in gene	Number of significant SNPs (p<0.05, r2<0.5)	Empirical gene-based p-value	List of significant SNP
CDK5	53	2	0.3147	rs4148853|rs34403003
IL1B	42	1	0.3147	rs3917381
ADAM10	232	1	0.3197	rs544282
NOS3	71	1	0.3197	rs12666075
NRG1	2784	5	0.3227	rs147179882|rs2466068|rs7829383|rs2347071|rs11998153
APBB1	119	1	0.3487	rs11040880
LRRK2	360	3	0.3526	rs189800607|rs10878411|rs11564173
OLIG2	123	2	0.3626	rs17632819|rs76708155
NOS1	559	5	0.3826	rs67313272|rs4767542|rs816284|rs12228022|rs10850829
HIF1A	82	1	0.4186	rs12891737
FGF1	221	5	0.4326	rs1808258|rs2070715|rs249925|rs10041541|rs13179022
TGFB1	131	1	0.5135	rs4803459
IGF1R	520	5	0.5325	rs11631965|rs4966039|rs3743254|rs7166348|rs2272037
FAS	175	1	0.5774	rs12767306
APP	554	5	0.6214	rs2829960|rs6516705|rs13046930|rs6516715|rs117104544
NTRK2	605	4	0.6264	rs17087710|rs28580203|rs1047896|rs1006446
DYRK1A	380	1	0.6593	rs28550863
ESR2	412	4	0.6983	rs1152576|rs4986938|rs10146107|rs12587140
RELN	1552	5	0.7932	rs2299373|rs3819491|rs39377|rs1476446|rs694894
ESR1	810	1	0.7972	rs55650062
ABCA2	61	0	1	NA
ADRA2A	27	0	1	NA
BDNF	122	0	1	NA
CAV1	129	0	1	NA
CDK5R1	24	0	1	NA
CHRNA7	2	0	1	NA
CNTF	29	0	1	NA
FOS	48	0	1	NA
GSK3B	329	0	1	NA
HTR2A	203	0	1	NA
NEUROD1	54	0	1	NA
NPY	131	0	1	NA
PLCG1	60	0	1	NA
PSEN1	154	0	1	NA
SIRT1	98	0	1	NA
THRA	82	0	1	NA
TNF	40	0	1	NA

Gene	Number of SNPs in gene	Number of significant SNPs (p<0.05, r2<0.5)	Empirical gene-based p-value	List of significant SNP
TNFR1A	4	0	1	NA
TNFR1B	54	0	1	NA
DLD	24	0	1	NA
GLP1R	98	0	1	NA
IFNG	19	0	1	NA
Plau	30	0	1	NA
PSEN2	19	0	1	NA
SOD2	5	0	1	NA
tp53	5	0	1	NA
CST3	92	0	1	NA
CHRM1	47	0	1	NA

Table 3

Association of rs9608282 in *ADORA2A* with neuroimaging phenotypes and memory composite scores with and without diagnosis (DX) adjustment. SNP-based association results (*p*-values) in the discovery sample for hippocampal volume, neurogenesis-related hippocampal sub-regions, memory performance, and CSF total tau level.

rs9608282	*p*-value after adjusting for DX	*p*-value without adjusting for DX
Hippocampal Volume	**3.29×10^{-4}**	**1.14×10^{-5}**
Neurogenesis-Related Hippocampal Sub-regions	**4.55×10^{-3}**	**2.50×10^{-4}**
Memory Composite Score	2.18×10^{-1}	**7.45×10^{-3}**
CSF Total Tau	2×10^{-1}	**2.3×10^{-2}**

Table 4

Meta-analysis of *ADORA2A* with hippocampal volume in three independent cohorts: ADNI, AddNeuroMed and IMAS.

	ADNI *p*-value	**AddNeuroMed *p*-value**	**IMAS *p*-value**	**Meta-analysis *p*-value**
ADORA2A	**5×10^{-5}**	2.1×10^{-1}	2.95×10^{-1}	**4.3×10^{-5}**

Table 5

Meta-analysis of rs9608282 with hippocampal volume in three independent cohorts: ADNI, AddNeuroMed and IMAS.

rs9608282	*N*	Effect of rs9608282 (T) (β value)	*p*-value
ADNI	1563	146.9	1.14×10^{-5}
AddNeuroMed	218	362.4	2.34×10^{-2}
IMAS	59	−263.2	4.59×10^{-2}
Meta-analysis	1840		$\mathbf{7.88\times10^{-6}}$